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Proton Inhibition of the NMDA-gated Channel in Isolated Catfish Cone Horizontal Cells

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The effect of H^+ on the *N*-methyl-D-aspartate-induced (NMDA) membrane current in enzymatically isolated catfish cone horizontal cells was investigated. Extracellular acidification to pH 5.5 blocked nearly completely the NMDA-induced current and reduced desensitization. The pK for the H^+ effect was 6.5, near that for the free amino acid histidine. Protons did not alter the receptor affinity for NMDA and the inhibition was insensitive to the membrane potential and surface charge screening. However, extracellular H^+ increased the IC_{50} for Zn^{2+}. These results indicate that protons can modulate the NMDA-induced membrane current by a mechanism that may include interaction with histidine residues. Copyright © 1996 Elsevier Science Ltd.

Catfish NMDA Patch-clamp Isolated horizontal cells Inhibition by hydrogen ion

INTRODUCTION

Membrane channel proteins that bind endogenous chemical agents are by their nature influenced by the extracellular environment. Many of these proteins have several modulator sites that regulate the ion permeation process. One of the most complex of this type of membrane protein is the *N*-methyl-D-aspartate (NMDA) receptor that is activated by the endogenous excitatory neurotransmitter L-glutamate. Normal constituents of the extracellular environment can modulate the NMDA receptor. Glycine acts as a co-agonist and magnesium as a voltage-dependent regulator of the NMDA receptor–channel complex. The NMDA receptor has an additional redox modulator site(s) (Aizenman *et al.*, 1989; Aizenman & Reynolds, 1992) that undergoes chemical modification by agents that alter sulfhydryls. In a completely reversible process, oxidation by 5,5-dithiobis-2-nitrobenzoic acid (DTNB), converting sulfhydryls to disulfides, decreases the NMDA-induced membrane current (Aizenman *et al.*, 1989). More recently, glutathione, a naturally occurring oxidizing agent (Slivka *et al.*, 1987; Kosower & Kosower, 1978) has been shown to mimic the effects of DTNB (Sucher & Lipton, 1991).

Agents that modify the native protein and pH titration are important tools for investigating those amino acid components necessary for the function of the membrane protein (Green & Andersen, 1991; Hille, 1992). Hydrogen ion titration has been widely used to alter the charge on membrane proteins in an attempt to provide information on specific amino acid residues important for the ion permeation process. This method has been effectively used to study the voltage-sensitive Na^+ (Begenisich & Danko, 1983; Sigworth, 1980; Woodhull, 1973; Hille *et al.*, 1975), K^+ (Hille, 1968) and Ca^{2+} (Prod'hom *et al.*, 1989) channels and agonist-gated nicotinic (Ben-Haim *et al.*, 1973; Lindstrom *et al.*, 1973; Landau *et al.*, 1981) and GABA channels (Gruol *et al.*, 1980). H^+ has been reported to inhibit the NMDA response in cerebellar (Traynelis & Cull-Candy, 1990, 1991) and hippocampal neurons (Tang *et al.*, 1990) and the kainate/quisqualate-gated channel in retinal horizontal cells (Christensen & Hida, 1990) and cerebellar neurons (Traynelis & Cull-Candy, 1991). Binding sites have been identified both inside and outside the channel pore, the location depending on the channel type. The effect of protons on all of the channels listed above is to decrease ion permeation. The mechanisms by which protons can antagonize the membrane current include blocking the channel, reducing fractional channel open time, reducing the open probability and reducing the single channel conductance. The change in extracellular pH occurring during the release of amino acid neurotransmitters may play an important role in the regulation of synaptic transmission (Chen & Chesler, 1992). In this study, we have examined the effect of protons on the NMDA-induced response in cone horizontal cells enzymatically isolated from the catfish retina (Hals *et al.*, 1986; O'Dell & Christensen, 1989). The reduction of the NMDA-induced membrane current by protons is believed to involve an interaction with histidine residues on the NMDA receptors that affects the transition from closed to open state. This is similar to the effect of protons on the non-NMDA receptors of this same cell type (Christensen & Hida, 1990). This conclusion is based, in part, on our

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data demonstrating that the pH at which 50% of the inhibition occurs is 6.5. This is near the pK for the imidazole group of the free amino acid histidine.

METHODS

Cell isolation procedure

The method of cell isolation has been detailed in a previous publication (O'Dell & Christensen, 1989). Briefly, Texas channel catfish (*Ictalurus punctatus*) were dark adapted for about 30 min. The fish were anesthetized in a solution of 3-aminobenzoic acid ethyl ester (1 mg/l; Sigma Chemical) until they no longer responded to noxious tactile stimuli. Following enucleation, both corneas and lenses were removed leaving an eyecup that was then incubated for 3 min in normal catfish saline (NCFS) (containing in mM: 126 NaCl, 4 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 16 dextrose and 10 Hepes, pH 7.4 adjusted with NaOH) and 0.5 mg/ml hyaluronidase to remove the vitreous. The retina was stripped from the eyecup, cut into 6–8 pieces, transferred to a beaker containing NCFS and stored at 4°C. Before isolation of the horizontal cells, a piece of the tissue was incubated in low-Ca^{2+} catfish saline (0.3 mM Ca^{2+}) containing 60 U/ml of cysteine-activated papain (Worthington Biochemical). The incubation time (normally 4–8 min) was adjusted according to the quality of the previously isolated cells. To obtain individual cells, following enzyme treatment, a piece of retina was mechanically dissociated by repeatedly passing the tissue through the tips of a series of fire polished Pasteur pipettes. The cells were plated in a saline-filled recording chamber mounted on the stage of a Nikon inverted phase-contrast microscope.

Recording

Recordings were made at room temperature (23°C). Isolated cells were voltage clamped (Iwazumi Mark II voltage clamp) using the single patch electrode in the whole-cell mode. Recording electrodes were pulled from 1.65 mm o.d. glass (Garner Glass Co.) on a Sutter model P-87 horizontal puller and filled with an electrode solution containing in mM: K-gluconate 140, $MgCl_2$ 2, BAPTA 2, $CaCl_2$ 0.2, HEPES 11, adjusted to pH 7.3 with KOH. In some experiments, CsCl was substituted for K-gluconate. Electrode resistance ranged from 1 to 5 MΩ. There was no series resistance or capacitance compensation. Solutions containing NMDA were made in Mg^{2+}-free NCFS supplemented with 1 μM glycine. To control extracellular pH, buffers were chosen for their optimum pH range: pH < 7.0, MES; pH 7.0–8.0, HEPES; pH > 8, TAPS (Sigma Chemical, St Louis, MO). Solution changes were made using a concentration–clamp system (O'Dell & Christensen, 1989). A high resistance seal between cell membrane and the patch pipette was formed by gently pressing the patch pipette on the cell surface and applying a small amount of suction to the pipette barrel. Rupture of the patch was achieved by applying a small brief negative voltage to the patch pipette under current clamp conditions. The amplifier was then switched to voltage clamp mode and the membrane potential was clamped to −65 mV. After rupture of the membrane patch, the cell was positioned in a gravity fed superfusion tube (approximate volume 100 μl). Rapid exchange of solution was achieved when a solenoid attached to the exit of the superfusion tube was opened siphoning new solution pipetted into an input well over the cell. At the fastest superfusion rates (3–4 ml/min), a complete solution change could be achieved in 20–40 msec.

Current–voltage relationships were constructed by subtracting the current activated by 10 mV voltage steps measured in the absence of agonist from currents measured at the same voltage in the presence of agonist. Concentration–response data were obtained by measuring the agonist-induced current in different concentrations of agonist. Each agonist application was followed by a rinse in agonist-free NCFS.

Measurement of intracellular pH

Isolated horizontal cells were loaded with the membrane permeant form of the pH-sensitive fluorescent dye BCECF-AM (Molecular Probes, Eugene, Ore.). Cells were incubated for 30 min in 5 μM BCECF-AM and 10% pluronic dissolved in DMSO. Cells were rinsed thoroughly in NCFS and plated in a chamber, mounted on a Nikon Diaphot microscope connected to a UV illumination system (Photon Technologies Inc.). The cells were alternately illuminated at 490 and 440 nm by a rotating chopper. Background fluorescence was measured from a BCECF-free cell and subtracted but the background signal was negligible. The change in the 490/440 nm ratio is indicative of the change in intracellular pH. Cells were not calibrated for absolute values of pH and only the change in ratio was measured.

RESULTS

H^+ *inhibits* N-*methyl-D-aspartate-induced response*

The membrane currents, recorded from a single horizontal cell voltage clamped at −65 mV were elicited by stepping the NMDA concentration to 100 μM using solutions in which the extracellular pH varied from 5.5 to 9.5 in steps of 0.5 pH units. Figure 1 illustrates the decrease in the NMDA-induced membrane current with changes in the extracellular $[H^+]$ (pH range 6.5–8.5). At pH 7.5, as illustrated in Fig. 1, the current response partially desensitizes. The time constant of the desensitization at pH 7.5, measured from cells in which desensitization was present, was 2.2 ± 1.2 sec (mean ± SD, $n = 6$). The magnitude as well as the time constants for desensitization were variable, with some cells showing little or no desensitization. In addition, a cell without a desensitizing response on first application of NMDA might have one during a second application. For these reasons, the desensitization process was difficult to study in these cells.

The effect of pH on desensitization was measured from the NMDA-induced membrane current from cells that

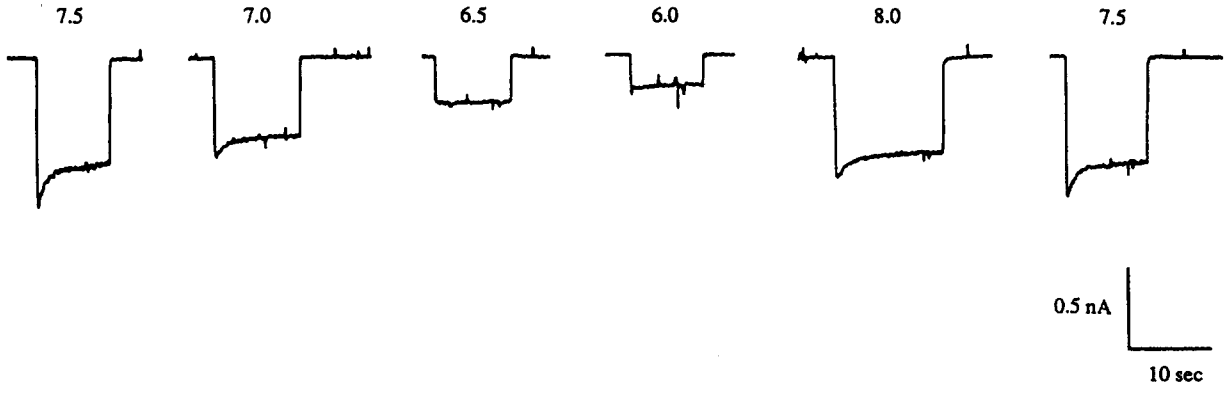

FIGURE 1. Whole-cell membrane current responses at different pHs (indicated above each trace) to 100 μM NMDA plus 10 μM glycine in Mg^{2+}-free solution applied under concentration-clamp conditions. Responses are from the same cell and were measured at steady-state. Cells were voltage clamped at −65 mV.

exhibited consistent desensitizing responses. The steady-state current in Fig. 1 was about 75% of the peak current at pH 7.5. The ratio of the peak to steady-state current shown in Fig. 2 illustrates that the hydrogen ion concentration probably has little or no effect on desensitization at pH > 7.5, since under these conditions the amount of desensitization did not change. However, when H^+ was increased, both the response and the desensitization decreased. Even when the current was amplified so that the initial current amplitude measured at pH 6.5 was the same as pH 7.5, no desensitization was apparent. Desensitization reappeared on return to pH 7.5. The initial control response to NMDA is fully recoverable if the cell does not remain for several minutes below pH 6.0.

Change in the pH_i does not affect the NMDA-induced response

It is well known that changing the extracellular pH affects the intracellular pH (Thomas, 1974; Aickin & Thomas, 1975; Deitmer & Ellis, 1980). Moreover, acidic intracellular pH has been shown to decrease responses to ACh (Goldberg & Lass, 1983). Acidification of the horizontal cell cytoplasm was produced by superfusing with NH_4Cl for several minutes. The acidification begins immediately on washing out the NH_4Cl (Boron & de Weer, 1976). This was confirmed in separate experiments using the pH-sensitive fluorescent dye 2',7-bis(2-carboxymethyl-5-(and-6)-carboxyfluorescine (BCECF). Periodic measurement (approximately every 30 sec) of the NMDA-induced current up to 30 min after exposure of the cell to NH_4Cl (30 mM) showed no change when compared to control currents taken before NH_4Cl (results not shown). We conclude that the inhibition of the NMDA response by low pH is due to the direct action of protons on the extracellular face of the NMDA receptor protein. This is identical to the results obtained for the non-NMDA receptors in this same cell type (Christensen & Hida, 1990).

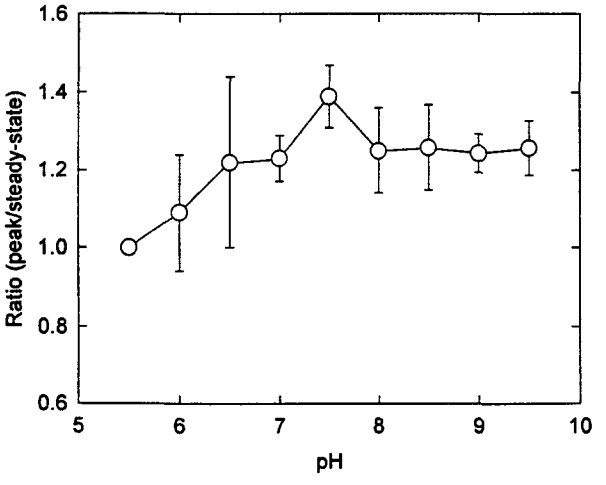

FIGURE 2. The effect of pH (abscissa) on receptor desensitization (ordinate, measured as the peak : steady-state current ratio). At pH > 7.4, there was no effect on desensitization. At pH < 7.4, the magnitude of desensitization decreased. Bars indicate SD measured from at least five cells at each point.

Since lowering the intracellular pH had no substantial effect on the NMDA-induced membrane current, the following experiments were designed to characterize the NMDA response when the extracellular pH was decreased.

Figure 3 shows a titration curve in which the NMDA-induced membrane current was plotted as a function of extracellular pH. The steady-state membrane currents were measured in 100 μM NMDA. The amplitude of the current measured at each pH was normalized to the membrane current measured at pH 7.4. A curve fit of concentration–response data was made using a modification of the Hill equation that included an offset. This is because even at the lowest pH used, there was a residual current measured in the presence of agonist, suggesting that a complete block of the NMDA-induced current by H^+ does not occur. The Hill coefficient was 1.3 ($\pm$ 0.082 SD, n = 7) suggesting cooperativity and possible multiple H^+ binding sites. The Hill coefficient was significantly different from 1 as determined from the Student's t-test ($P < 0.001$, two tailed t-test). The pK (6.45, SD 0.09, n = 7) was near the pK of the imidazole group of the free amino acid histidine. The Hill coefficient and the pK obtained by fitting the normalized peak current (Hill coefficient, 1.36 SD $\pm$ 0.1; pK, 6.46 SD $\pm$ 0.1, n = 7) was near that obtained from a fit of the steady-state current. It might be expected that the slope of the titration

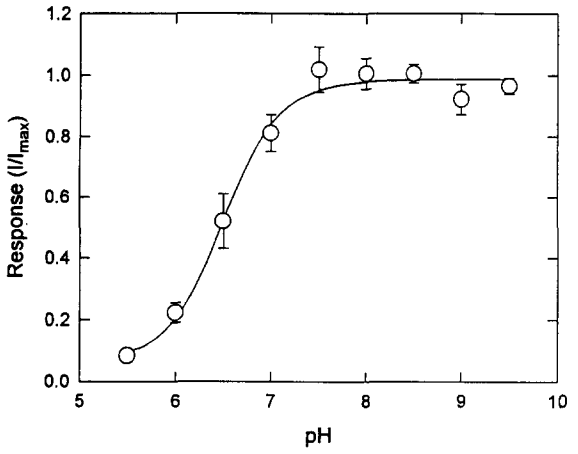

FIGURE 3. The titration curve of the pH inhibition effect. Responses to 100 μM NMDA plus 10 μM glycine were normalized to the maximum response (I_{max}) measured at steady-state over the pH range 5.5–9.5. The smooth curve represents the curve fit of the data using the Hill equation of the form

$$I = I_{res} + \frac{(I_{max} - I_{res})[pH]^n}{[pK]^n + [pH]^n}$$

where I_{max} is the current at pH 7.5, I_{res} is the membrane current which cannot be inhibited by protons; n is the slope of the curve (Hill coefficient) and pK is the pH when the current is 50% of the maximum. The parameters from the fitted curve were: pK = 6.45; n = 1.3; I_{res} = 0.08. A complete titration curve was measured from each cell. The number of observations was seven. Bars indicate SD.

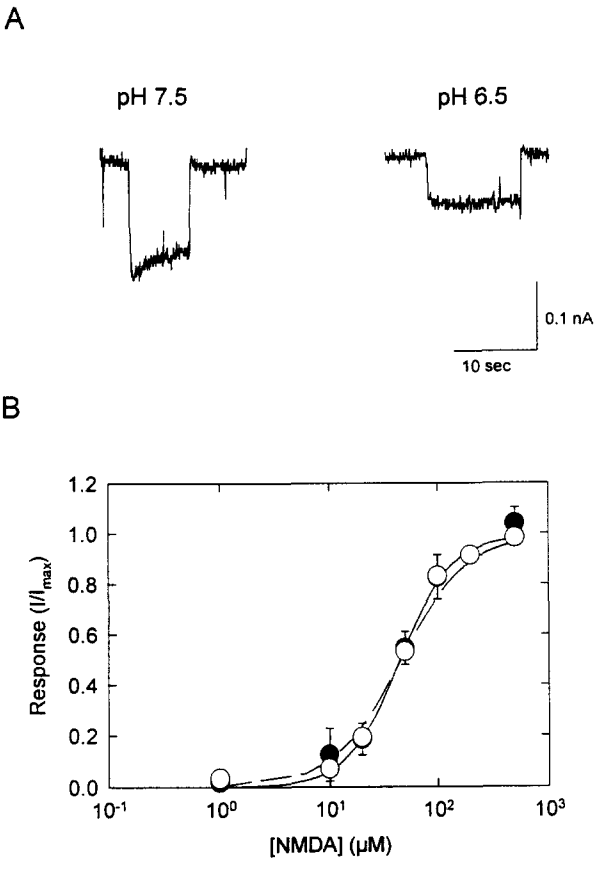

FIGURE 4. (A) Traces representing the NMDA-induced membrane current recorded at pH 7.5 and 6.5 in 500 μM NMDA measured from the same cell. (B) Concentration–response curve for peak NMDA response at pH 7.5 and 6.5. Both sets of data points were obtained from the same seven cells, first at pH 7.5 (○) and then pH 6.5 (●). The data sets are normalized to I_{max}. Each data set was curve fit using the Hill equation of the form

$$I = \frac{I_{max}[A]^n}{[EC_{50}]^n + [A]^n}$$

where I_{max} is the current at saturating concentrations of agonist [A], EC_{50} is the current at one-half the I_{max} and n is the Hill coefficient. The smooth curves are fits to each data set. The values obtained from the curve fits are listed in Table 1. Bars indicate SD.

curve would be steeper from the data using the peak current compared to the current measured at steady-state. In five of the seven cells used for these experiments, the Hill coefficient was larger measured from the peak current data set as determined from the individual fitted curves. In the other two cells the Hill coefficients were identical. We used the paired t-test to determine if the Hill coefficients or the pKs were significantly different. However, at $P = 0.5$ level, the difference was not great enough to exclude the possibility that it was due entirely to chance.

H^+ *inhibits the NMDA response by a noncompetitive mechanism*

One mechanism explaining the effect of protons on the NMDA-induced response would be competition for the NMDA receptor binding site. Under conditions of competitive inhibition, the EC_{50} would shift to the higher agonist concentration in the presence of protons but the maximum response would not change. By comparison, under conditions of noncompetitive inhibition, the EC_{50} would not change but the maximum response at a saturating concentration of agonist decreases. These two possibilities were tested by measuring NMDA concentration–response curves from whole cell currents at acidic (pH 6.5) and normal pH (pH 7.5). Figure 4(A) shows the NMDA-induced response at a saturating concentration of the agonist from the same cell at pH 7.5 and 6.5. The decrease in the maximum NMDA response at pH 6.5 and the unchanged EC_{50} shown in the concentration–response curves in Fig. 4(B) clearly indicate that protons decreased the membrane current by a mechanism of noncompetitive inhibition. The parameters I_{max} and EC_{50} and Hill coefficient, at each pH obtained from the fitted curves are indicated in Table 1.

H^+ *antagonism is voltage independent*

The blocking of NMDA current by H^+ may simply be due to interaction with the binding site(s) for permeant

TABLE 1. Effect of pH on the NMDA receptor properties

	pH 7.5	pH 6.5
Hill coefficient	1.42	1.8
I_{max}	0.9 ± 0.15 nA	0.45 ± 0.13 nA
EC_{50}	46 μM	45 μM

This table summarizes the characteristics of the catfish horizontal cells to NMDA at pH 7.5 and 6.5. For each parameter the results were obtained from 7–12 cells.

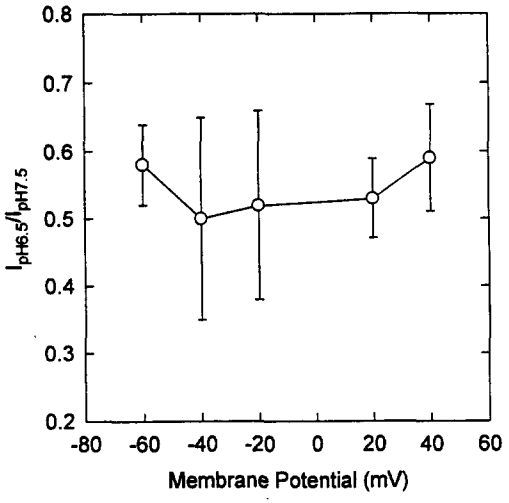

FIGURE 5. Ratio of the current recorded at pH 6.5 divided by the current recorded at pH 7.5 over the range of membrane potential from −65 to +40 mV. The vertical bars represent the SD for each determination. The concentration of NMDA at both pHs was 100 μM. The number of cells was five.

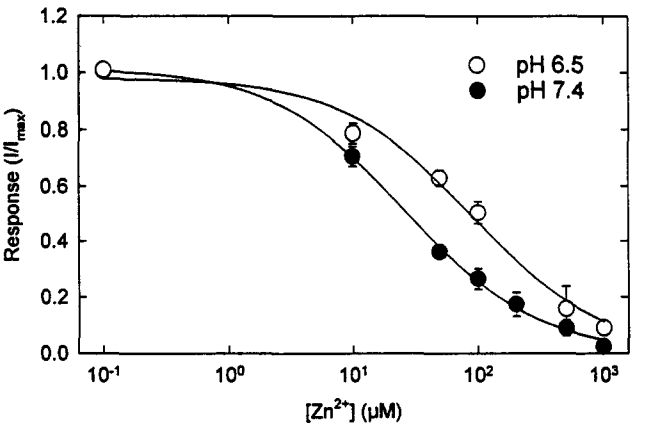

FIGURE 6. Zn^{2+} inhibition concentration–response curves measured at pH 6.5 and 7.4. The IC_{50} for Zn^{2+} was measured at pH 7.4 to be 26.4 μM and shifted to 89 μM at pH 6.5. See text for details.

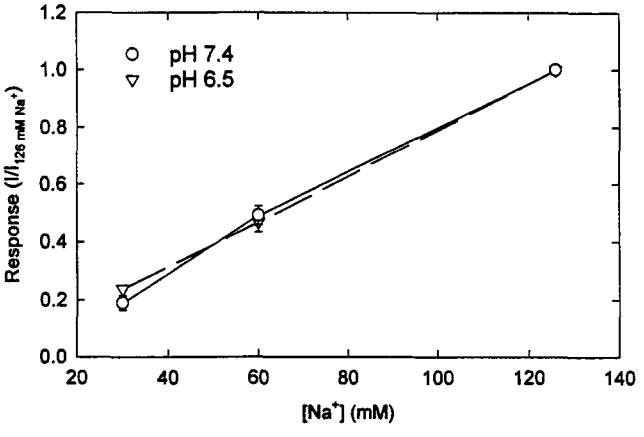

FIGURE 7. The effect of altering surface charge on NMDA-induced membrane current. The NMDA (100 μM plus 10 μM glycine) responses at pH 6.5 (▽) and pH 7.4 (○) were measured at three different ionic conditions: $[Na^+]_o$ 30, 60 and 126 mM, respectively. NMDA replaced Na^+ to maintain osmolarity of the saline at 270 mosm. Bars indicate SD.

ions as they pass through the channel pore. If the site is within the transmembrane electrical field, this type of block always shows a dependence on membrane potential (Woodhull, 1973). Figure 5 shows the ratio of the NMDA-induced current measured at pH 6.5 and pH 7.5 plotted as a function of voltage. The membrane current was measured over a range of membrane potential from −60 to +40 mV. If the inhibition of the membrane current at pH 6.5 were relieved by depolarizing the cell membrane potential as expected for a voltage-dependent block, the ratio should approach 1 at positive potentials. As shown in Fig. 5, the membrane current is reduced uniformly over the whole range of voltages. This result suggests that if protons do block the channel, they do so from a position outside the membrane electrical field. When the data were plotted as traditional current–voltage curves, there was no shift in the reversal potential, consistent with the hypothesis that H^+ does not significantly permeate the channel (although the change in H^+ concentration may be too small to elicit an observable shift in the reversal potential in the face of highly permeant monovalent and divalent cations).

Protons reduce the affinity of Zn^{2+} for its binding site

Several modulation sites have been identified for the NMDA receptor (Wong & Kemp, 1991; Mayer *et al.*, 1988; Johnson & Ascher, 1987). They include sites for the organic modulators glycine and polyamines and inorganic divalent ions Mg^{2+} and Zn^{2+}. None of these modulators compete with the binding of the agonist to the receptor. In separate experiments, interactions between protons and these modulators were investigated. Only the binding of Zn^{2+} was affected by the presence of protons.

Zn^{2+} has been shown to inhibit the NMDA response by a noncompetitive mechanism (Westbrook & Mayer, 1987). As shown in Fig. 6, the inhibition curve for Zn^{2+} on the response to NMDA was shifted to the right when the pH was decreased from 7.4 to 6.5, indicating a reduction in the apparent affinity of Zn^{2+} for its binding site. The smooth lines are the curve fits using the Hill equation. The IC_{50}s measured from the fitted curves were 89 μM at pH 6.5 and 26.4 μM at pH 7.5.

H^+ does not inhibit the NMDA response by screening surface charge

Due to the negative surface potential resulting from negatively charged amino acids on the extracellular face of the channel protein or from phospholipids, the cation concentration near the membrane is higher than that found in the bulk solution. This surface charge can be neutralized by substituting impermeant for permeant cations. This would result in a decrease in current, since the single channel conductance is generally dependent on the ionic concentration (Green & Andersen, 1991). Figure 7 shows the results from experiments designed to test if protons could screen surface charge sufficiently to reduce the membrane current. The membrane current was measured at lower ionic strength, because under

these conditions it is easier to observe the dependence of channel conductance on the ionic concentration. The NMDA response was measured at normal (126 mM Na^+) and two reduced Na^+ (60 and 30 mM) concentrations in both normal (pH 7.4) and increased H^+ (pH 6.5). Figure 7 shows the ratio of the NMDA-induced current measured in reduced and in normal $[Na^+]_o$ plotted against the $[Na^+]_o$. If H^+ titrates surface charge to the extent that the local ionic activity is affected, then the NMDA current obtained at reduced ionic strength would decrease more at pH 6.5 than at normal pH. The results for two different pHs do not differ, indicating that H^+ does not inhibit the NMDA response by neutralizing the negative charges on the receptor protein.

DISCUSSION

NMDA has three pK values similar to those of aspartate (α-COOH, 1.88; β-COOH, 3.65; α-NH_3^+, 9.6). Since these pKs are far from the pH range used in these experiments, it is unlikely that the observed depression of the NMDA-induced current was due to titration of the NMDA molecule itself.

H^+ has an almost universal inhibitory effect on all cation permeable surface membrane channels. In the CNS, excitatory amino acids activate two types of membrane channels that can be generally classified as NMDA and non-NMDA. The non-NMDA glutamate channels found in both catfish cone horizontal cells (Christensen & Hida, 1990) and cerebellar neurons (Tang *et al.*, 1990; Traynelis & Cull-Candy, 1991) are both inhibited by H^+, with pKs between 5.7 and 6.5. Interestingly, non-NMDA glutamate channels in embryonic rat hippocampus were unaffected by H^+ (Tang *et al.*, 1990) although the NMDA activated channels were inhibited at acidic pH and potentiated at alkaline pH. The mechanism of H^+ inhibition of the NMDA response is not the same for different neuronal cell types. Traynelis and Cull-Candy (1991) have published the most comprehensive study demonstrating the effects of H^+ on the NMDA-induced membrane current. In the discussion that follows, we emphasize comparisons between our results on horizontal cells and those reported by Traynelis and Cull-Candy (1991) on rat cerebellar granule cells.

The pK for H^+ inhibition of the NMDA-induced membrane current in horizontal cells is 6.5, which is similar to the pK for the non-NMDA receptor on the same cell type (Christensen & Hida, 1990). In contrast, the pK for H^+ inhibition of NMDA-induced currents in cerebellar granule cells is 7.3, well within the physiological range of extracellular pH, suggesting that the NMDA response is normally nearly half inhibited at physiological pH (Traynelis & Cull-Candy, 1991). However, in the same cells, the pK for the H^+ inhibition of the kainate-induced membrane current is 5.7, and the pK is 6.3 for the quisqualate-induced current (Traynelis & Cull-Candy, 1991). These differences may simply reflect protein folding within the membrane in ways that adjacent polar or non-polar groups can differentially influence the pK of the titratable amino acid groups.

The single channel conductance of the non-NMDA receptors in these cells ranges from 5 to 12 pS measured from current fluctuations (O'Dell & Christensen, 1989) and directly from single channel measurements (Lasater, 1990). In mammalian neurons, the conductance of the NMDA channel lies between 25 and 50 pS (Nowak *et al.*, 1984; Westbrook *et al.*, 1986; O'Brien & Fischbach, 1986; Jahr & Stevens, 1987; Cull-Candy & Usowicz, 1987; Traynelis & Cull-Candy, 1991). Although lower conductance states (near 10 pS) have been reported for mammalian neurons (Cull-Candy *et al.*, 1988; Howe *et al.*, 1991), these are believed to be due to non-specific activation of non-NMDA receptors (Traynelis & Cull-Candy, 1991). We have measured a conductance for the NMDA-gated channel from current fluctuation of near 5 pS (O'Dell & Christensen, 1989). The lower conductance of the NMDA activated channels in catfish horizontal cells may reflect a real difference in subunit structure. Although we have not measured the single channel conductance of the NMDA-gated channel at low pH, the evidence from cerebellar neurons suggests that channel conductance is not affected by pH (Traynelis & Cull-Candy, 1991).

H^+ *acts extracellularly at a site separate from the NMDA or glycine binding sites*

Our results show that an increase in intracellular H^+ did not affect the NMDA response. It takes about 20 min for intracellular pH to change from 7.3 to 7.1 in snail neurons when extracellular pH is changed from 7.5 to 6.0 (Thomas, 1974). This is too slow for an intracellular change in pH to be responsible for the inhibition following an increase in extracellular H^+ in our experiments, because the effect of pH was observed immediately upon exposure of the cell to NMDA at the lower pH.

Glycine is believed to be a co-agonist with NMDA at the channel protein. Therefore, an attractive hypothesis is that H^+ competes with glycine for its binding site. The pK of the proton inhibition is about 6.5 giving an IC_{50} of about 300 nM H^+. The EC_{50} for glycine is about 185 nM (Mayer *et al.*, 1989a). The reversal of the pH inhibitory effect in the presence of a high concentration of glycine would implicate the glycine binding site. However, 400 μM glycine did not affect the steady-state current at pH 6.5. This is in contrast to the observation by Tang *et al.* (1990), who described a greater effect of H^+ at lower glycine concentrations, but is consistent with the results obtained by Traynelis and Cull-Candy (1991) who reported only a small proton-dependent effect on glycine. In addition, protons act at a site separate from the NMDA receptor binding site, based on the observation that protons reduce the maximum NMDA response with little effect on the EC_{50}. A similar conclusion was reached by Traynelis and Cull-Candy (1991) and Tang *et al.* (1990).

Unlike the results reported by Traynelis and Cull-Candy (1990), we observed a marked effect of pH on desensitization. The desensitizing NMDA-induced current in horizontal cells is quite labile. Some cells do not desensitize even with rapid (< 50 msec) applications of

agonist. Other cells show small desensitizing responses. In those cells in which desensitization was observed, it disappeared in the presence of H^+. However, reduction of desensitization cannot account for the decrease in NMDA-induced membrane current because desensitization accounts for less than 10% of the response.

A decrease in channel open time is one possible model that explains the effect of H^+. This might involve a process whereby protonation reduces the energy barrier for channel closing possibly by changes in the local electrostatic interactions of charged groups without significant change in the protein conformation (Hurley *et al.*, 1990). At present we cannot discount this possibility.

In this model the channel is unprotonated at pH > 7.5 and the apparent open time reflects that of the unprotonated channel. As the pH is progressively reduced, more channels become protonated and the measured apparent open time is the weighted average of protonated and unprotonated channels. This model implies two types of channels, one with a long open time (unprotonated channel) and one with a shorter open time (protonated channel). However, the on and off rate constants for the reaction of H^+ with its substrate are likely to be fast, relative to the opening and closing of the channel. This model then predicts that at any pH, at equilibrium, one would observe only one open state whose apparent open time would be dependent on the concentration of H^+. There is a second piece of evidence that supports a decrease in channel open time but is inconsistent with a mechanism in which complete block of the receptor/channel complex occurs. At the lowest pH tested (5.5) a residual current persists in the presence of agonist. Using the Hill equation plus an offset to fit the current–pH curve (Fig. 3), the low pH asymptote predicts 8% of the maximum current remaining. This suggests that any mechanism, such as competition for the agonist receptor site or channel block, that would completely eliminate the membrane current is unlikely, whereas a mechanism that reduces open time to a small but finite value will do so maximally under conditions in which all open channels are fully protonated. In the fully protonated condition, a small residual current would still be observed.

H^+ *reduces the affinity of* Zn^{2+} *for its binding site*

Our results show that H^+ can reduce the affinity of Zn^{2+} for its binding site on NMDA receptors. Our data cannot distinguish between competitive, allosteric interference or surface charge screening mechanisms. However, it is not unique for divalent ions to compete with monovalent ions for the binding sites. Ca^{2+} and Ba^{2+} can compete with H^+ for a binding site(s) in the calcium channel of muscle cells (Prod'hom *et al.*, 1987). Spires and Begenisich (1992) have reported a similar case in which they showed that Zn^{2+} competes with H^+ for a binding site in inhibiting K^+ current of stellate ganglion giant fiber lobe cells of squid. Although Zn^{2+} has been shown to bind to sulfhydryls, it does not bind to sulfhydryls in inhibiting the NMDA response (Mayer *et al.*, 1989b). Therefore, it is possible that Zn^{2+} and H^+ compete for the imidazole group of the histidyl residue.

Because the pK for inhibition is near that for the free amino acid histidine, it is tempting to conclude that histidine residues form part of the gate of the NMDA channel in catfish cone horizontal cells and that the protonation of the gate regulates how easily it can overcome the energy barrier that prevents closing. Other evidence supports the hypothesis that histidine residues may be involved in the H^+ regulation of the NMDA-gated channel in catfish cone horizontal cells (Wu and Christensen, submitted). The histidine modifying agents diethylpyrocarbonate (DEPC) and Rose bengal both inhibit the NMDA-induced membrane current. The rate of NMDA receptor modification by DEPC is pH dependent and is slower at lower pH under conditions in which the imidazole group would be protonated. The pK for the rate of DEPC modification is also 6.5, consistent with protonation of a histidine residue.

REFERENCES

Aickin, C. C. & Thomas, R. C. (1975). Micro-electrode measurement of the internal pH of crab muscle fibers. *Journal of Physiology, 252*, 803–815.

Aizenman, E., Lipton, S. A. & Loring, R. H. (1989). Selective modulation of NMDA response by reduction and oxidation. *Neuron, 2*, 1257–1263.

Aizenman, E. & Reynolds, I. J. (1992). Modulation of NMDA excitotoxicity by redox reagents. *Annals of the New York Academy of Science, 648*, 125–131.

Begenisich, T. B. & Danko, M. J. (1983). Hydrogen ion block of sodium pore in squid giant axons. *Journal of General Physiology, 82*, 599–618.

Ben-Haim, D., Landau, E. M. & Silman, I. (1973). The role of a reactive disulfide bond in the function of the acetylcholine receptor at the frog neuromuscular junction. *Journal of Physiology, 234*, 305–325.

Boron, W. F. & de Weer, P. D. (1976). Intracellular pH transients in squid giant axons caused by CO_2, NH_3, and metabolic inhibitors. *Journal of General Physiology, 67*, 91–112.

Chen, J. C. T. & Chesler, M. (1992). Modulation of extracellular pH by glutamate and GABA in rat hippocampal slices. *Journal of Neurophysiology, 67*, 29–36.

Christensen, B. N. & Hida, E. (1990). Protonation of histidine groups inhibits gating of the quisqualate/kainate channel protein in isolated catfish cone horizontal cells. *Neuron, 5*, 471–478.

Cull-Candy, S. G., Howe, J. R. & Ogden, D. C. (1988). Noise and single channels activated by excitatory amino acids in rat cerebellar granule neurones. *Journal of Physiology, 400*, 189–222.

Cull-Candy, S. G. & Usowicz, M. M. (1987). Multiple-conductance channels activated by excitatory amino acids in cerebellar neurons. *Nature, 325*, 525–528.

Deitmer, J. W. & Ellis, D. (1980). Interaction between the regulation of intracellular pH and sodium activity of sheep cardiac Purkinje fibres. *Journal of Physiology, 304*, 471–488.

Goldberg, G. & Lass, Y. (1983). Evidence for acetylcholine receptor block by intracellular hydrogen ions in cultured chick myoballs. *Journal of Physiology, 343*, 429–437.

Green, W. N. & Andersen, O. S. (1991). Surface charges and ion channel function. *Annual Review of Physiology, 53*, 341–359.

Gruol D. L., Barker, J. L., Huang, L-Y. M., MacDonald, J. F. & Smith, T. G. Jr (1980). Hydrogen ions have multiple effects on the excitability of cultured mammalian neurons. *Brain Research, 183*, 247–252.

Hals, G., Christensen, B. N., O'Dell, T., Christensen, M. & Shingai, R.

(1986). Voltage-clamp analysis of currents produced by glutamate and some glutamate analogues on horizontal cells isolated from the catfish retina. *Journal of Neurophysiology, 56*, 19–31.

Hille, B. (1968). Charges and potentials at the nerve surface: Divalent ions and pH. *Journal of General Physiology, 51*, 231–236.

Hille, B. (1992). *Ionic channels of excitable membranes* (pp. 390–393). Sunderland, MA.: Sinauer Associates Inc.

Hille, B., Woodhull, A. M. & Shapiro, B. I. (1975). Negative surface charge near sodium channels of nerve: Divalent ions, monovalent ions, and pH. *Philosophical Transactions of the Royal Society of London, 270*, 301–318.

Howe, J. R., Cull-Candy, S. G. & Colquhoun, D. (1991). Currents through single glutamate receptor channels in outside-out patches from rat cerebellar granule cells. *Journal of Physiology, 432*, 143–202.

Hurley, J. H., Dean, A. M., Thorsness, P. E., Koshland, D. E. Jr & Stroud, R. M. (1990). Regulation of isocitrate dehydrogenase by phosphorylation involves no long-range conformational change in the free enzyme. *Journal of Biological Chemistry, 265*, 3599–3602.

Jahr, C. E. & Stevens, C. F. (1987). Glutamate activates multiple single channel conductances in hippocampal neurons. *Nature, 325*, 522–525.

Johnson, J. W. & Ascher, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature, 325*, 529–531.

Kosower, N. S. & Kosower, E. M. (1978). The glutathione status of cells. *International Review of Cytology, 54*, 109–160.

Landau, E. M., Gavish, B., Nachshen, D. A. & Lotan, I. (1981). pH dependence of the acetylcholine receptor channel: A species variation. *Journal of General Physiology, 77*, 647–666.

Lasater, E. M. (1990). Properties of non-NMDA excitatory amino acid-activated channels in isolated retinal horizontal cells. *Journal of Neuroscience, 10*, 1654–1663.

Lindstrom, J. M., Singer, S. J. & Lennos, E. S. (1973). The effects of reducing and alkylating agents on the acetylcholine receptor activity of frog sartorius muscle. *Journal of Membrane Biology, 11*, 217–226.

Mayer, M. L., Vyklicky, L. Jr & Clements, J. (1989a). Regulation of NMDA receptor desensitization in mouse hippocampal neurons by glycine. *Nature, 338*, 425–427.

Mayer, M. L., Vyklicky, L. Jr & Westbrook, G. L. (1989b). Modulation of excitatory amino acid receptors by group IIB metal cations in cultured mouse hippocampal neurones. *Journal of Physiology, 415*, 329–350.

Mayer, M. L., Westbrook, G. L. & Vyklicky, L. Jr (1988). Sites of antagonist action on *N*-methyl-D-aspartic acid receptors studied using fluctuation analysis and a rapid perfusion technique. *Journal of Neurophysiology, 60*, 645.

Nowak, L., Bregestovski, P., Ascher, P., Herbet, A. & Prochiantz, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature, 307*, 462–465.

O'Brien, R. J. & Fischbach, G. G. (1986). Characterization of excitatory amino acid receptors expressed by embryonic chick motoneurons *in vitro*. *Journal of Neuroscience, 6*, 3275–3283.

O'Dell, T. J. & Christensen, B. N. (1989). Horizontal cells isolated from catfish retina contain two types of excitatory amino acid receptors. *Journal of Neurophysiology, 61*, 1097–1109.

Prod'hom, B., Pietrobon, D. & Hess, P. (1987). Direct measurement of proton transfer rates to a group controlling the dihydropyridine-sensitive Ca^{2+} channel. *Nature, 329*, 243–246.

Prod'hom, B., Pietrobon, D. & Hess, P. (1989). Interactions of protons with single open L-type calcium channels. Location of protonation site and dependence of protein-induced current fluctuations on concentration and species of permeant ion. *Journal of General Physiology, 94*, 23–42.

Sigworth, F. J. (1980). The conductance of sodium channels under conditions of reduced current at the node of Ranvier. *Journal of Physiology, 307*, 131–142.

Slivka, A., Spina, M. B. & Cohen, G. (1987). Reduced and oxidized glutathione in human and monkey brain. *Neuroscience Letters, 74*, 112–118.

Spires, S. & Begenisich, T. (1992). Chemical properties of the divalent cation binding site on potassium channels. *Journal of General Physiology, 100*, 181–193.

Sucher, N. J. & Lipton, S. A. (1991). Redox modulatory site of the NMDA receptor–channel complex: Regulation by oxidized glutathione. *Journal of Neuroscience Research, 30*, 582–591.

Tang, C. M., Dichter, M. & Morad, M. (1990). Modulation of the *N*-methyl-D-aspartate channel by extracellular H^+. *Proceedings of the National Academy of Science USA, 87*, 6445–6449.

Thomas, R. C. (1974). Intracellular pH of snail neurons measured with a new pH-sensitive glass micro-electrode. *Journal of Physiology, 238*, 159–180.

Traynelis, S. F. & Cull-Candy, S. G. (1990). Proton inhibition of *N*-methyl-D-aspartate receptors in cerebellar neurons. *Nature, 345*, 347–350.

Traynelis, S. F. & Cull-Candy, S. G. (1991). Pharmacological properties and H^+ sensitivity of excitatory amino acid receptor channels in rat cerebellar granule neurones. *Journal of Physiology, 433*, 727–763.

Westbrook, G. L. & Mayer, M. L. (1987). Micromolar concentrations of Zn^{2+} antagonize NMDA and GABA responses of hippocampal neurons. *Nature, 328*, 640–643.

Westbrook, G. L., Mayer, M. L., Namboidiri, M. A. A. & Neale, J. H. (1986). High concentrations of *N*-actylaspartrylglutamate (NAAG) selectively activate NMDA receptors on mouse spinal cord neurons in cell culture. *Journal of Neuroscience, 6*, 3385–3392.

Wong, E. H. F. & Kemp, J. A. (1991). Sites for antagonism on the *N*-methyl-D-aspartate receptor channel complex. *Annual Review of Pharmacology and Toxicology, 31*, 401–425.

Woodhull, A. M. (1973). Ionic blockage of sodium channels in nerve. *Journal of General Physiology, 61*, 687–708.

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